A Link between Aurora Kinase and Clp1/Cdc14 Regulation Uncovered by the Identification of a Fission Yeast Borealin-Like Protein

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The chromosomal passenger complex (CPC) regulates various events in cell division. This complex is composed of a catalytic subunit, Aurora B kinase, and three nonenzymatic subunits, INCENP, Survivin, and Borealin. Together, these four subunits interdependently regulate CPC function, and they are highly conserved among eukaryotes. However, a Borealin homologue has never been characterized in the fission yeast, *Schizosaccharomyces pombe*. Here, we isolate a previously uncharacterized *S. pombe* protein through association with the Cdc14 phosphatase homologue, Clp1/Flp1, and identify it as a Borealin-like member of the CPC. Nbl1 (novel Borealin-like 1) physically associates with known CPC components, affects the kinase activity and stability of the *S. pombe* Aurora B homologue, Ark1, colocalizes with known CPC subunits during mitosis, and shows sequence similarity to human Borealin. Further analysis of the Clp1–Nbl1 interaction indicates that Clp1 requires CPC activity for proper accumulation at the contractile ring (CR). Consistent with this, we describe negative genetic interactions between mutant alleles of CPC and CR components. Thus, this study characterizes a fission yeast Borealin homologue and reveals a previously unrecognized connection between the CPC and the process of cytokinesis in *S. pombe*.

INTRODUCTION

To ensure successful cell division, sister chromatids must segregate to opposite poles of dividing cells in mitosis and be partitioned into two new daughter cells during cytokinesis (Pines and Rieder, 2001). Highly intricate mechanisms control these processes, with various macromolecular complexes coordinating their activities such that the integrity of cell division is maintained. The chromosomal passenger complex (CPC), which is composed of a catalytic subunit, Aurora B kinase, and three nonenzymatic subunits, INCENP, Survivin, and Borealin, functions as one of these critical regulatory complexes. As its name implies, this complex travels on chromosomes to various sites during cell division such that it can execute specific tasks at distinct locations and times (Earnshaw and Bernat, 1991). These functions include, but are not limited to, chromosome condensation, stabilization of the mitotic spindle, correction of

improper kinetochore-microtubule attachments, and regulation of cytokinesis (for reviews, see Vagnarelli and Earnshaw, 2004; Vader *et al.*, 2006; Ruchaud *et al.*, 2007).

The four CPC subunits are highly interdependent for proper localization, activity, and stability (Ruchaud *et al.*, 2007). Consistent with such interdependency, these subunits are widely conserved among eukaryotes (Ruchaud *et al.*, 2007). However, it has proven difficult to identify Borealin homologues in yeasts. Though some have speculated that yeast Borealin homologues might not exist because of fusion of the Borealin and Survivin subunits into a single yeast Survivin homologue (Vader *et al.*, 2006), recent evidence suggests that there are in fact yeast Borealin homologues (Nakajima *et al.*, 2009). Nonetheless, a Borealin homologue in the fission yeast, *Schizosaccharomyces pombe*, has not been characterized.

Similar to the CPC, Cdc14-family phosphatases are also significant regulators of cell division. Named for Cdc14, the founding member identified in the budding yeast, *Saccharomyces cerevisiae*, these phosphatases reverse Cdk-mediated phosphorylation events to promote mitotic exit and cytokinesis (Queralt and Uhlmann, 2008). Like other Cdc14 family members, the *S. pombe* Cdc14 homologue, Clp1/Flp1, also functions in these processes. Specifically, Clp1 reverses Cdk1-mediated phosphorylation of the mitotic inducer Cdc25, allowing degradation of Cdc25 by the anaphase-promoting complex/cyclosome at the end of mitosis and ending the Cdk1 auto-amplification loop (Esteban *et al.*, 2004; Wolfe and Gould, 2004). Furthermore, Clp1 associates with the contractile ring (CR) scaffold protein Mid1, and, through this inter-

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Abbreviations used: BF, bright field; CPC, chromosomal passenger complex; CR, contractile ring; LatA, latrunculin A.

action, Clp1 enhances CR stability and the precision of cytokinesis (Clifford *et al.*, 2008).

In addition to these crucial functions during the concluding stages of the cell cycle, Cdc14-family phosphatases regulate chromosome segregation and CPC function during mitosis. In *S. pombe*, Clp1 associates with the *S. pombe* Aurora B kinase homologue, Ark1, and deletion of *clp1* results in increased cosegregation of sister chromatids (Trautmann *et al.*, 2004). In *S. cerevisiae*, Cdc14 regulates localization of the CPC to the spindle during anaphase (Pereira and Schiebel, 2003; Stoepel *et al.*, 2005). This Cdc14-mediated targeting of the CPC is crucial to its subsequent recruitment of the separase Esp1 and the chromosomal passenger Slk19 (Pereira and Schiebel, 2003; Khmelinskii *et al.*, 2007) that influence the formation and stabilization of the mitotic spindle and the spindle midzone (Zeng *et al.*, 1999; Jensen *et al.*, 2001). Yet, although it is clear that Cdc14-family phosphatases regulate CPC localization and function, a reciprocal relationship has not been described.

Though effects of the CPC on Cdc14-family phosphatases are unclear, the CPC is known to play a critical role in cytokinesis in many organisms (Carmena, 2008). For example, in *S. cerevisiae*, CPC subunits regulate septin dynamics (Gillis *et al.*, 2005; Thomas and Kaplan, 2007), and the Aurora B homologue, Ipl1, mediates a NoCut checkpoint that delays cytokinesis when chromatin is stalled in the cleavage plane (Norden *et al.*, 2006; Mendoza *et al.*, 2009). Interestingly, Aurora B functions in a similar checkpoint in human cells, inhibiting abscission in the presence of unsegregated DNA (Steigemann *et al.*, 2009). However, in *S. pombe*, the contribution of the CPC to cytokinesis is thought to be negligible. Overexpression of kinase-dead Ark1 does not affect cytokinesis but instead results in cut phenotypes in which septa form through unsegregated DNA (Petersen and Hagan, 2003), and *ark1* temperature-sensitive mutants similarly exhibit cut phenotypes. Because the division machinery appears intact in cut cells, these data suggest that Ark1 does not play a critical role in *S. pombe* cytokinesis (Petersen and Hagan, 2003). As a result, connections between the CPC and the *S. pombe* cytokinetic apparatus have not been further explored.

Through our studies of Clp1-associated proteins in *S. pombe* that will be discussed in detail elsewhere, we identified a Borealin-like protein, Nbl1. Here, we characterize Nbl1 as an *S. pombe* Borealin homologue by describing its CPC associations, its relevance to Ark1 activity and stability, its localization during the cell cycle, and its sequence and proposed structural similarity to human Borealin. Our analysis of the Nbl1-Clp1 association reveals that CPC activity is required for proper accumulation of Clp1 at the CR. We also present additional genetic evidence that the CPC influences cytokinesis. This study confirms the presence of a Borealin homologue in *S. pombe* and provides data linking the CPC and the process of cytokinesis in *S. pombe*.

MATERIALS AND METHODS

Strains and General Yeast Methods

The *S. pombe* strains used in this study (Supplementary Table 1) were grown in either yeast extract (YE) media or Edinburgh minimal media (EMM) with relevant supplements. *nbl1*(1-95), *nbl1*, *bir1*, *pic1*, *ark1*, *sid4*, *nog1*, *nuf2*, and *clp1* were tagged endogenously at the 3' end with 3XFLAG:hyg^R, GFP:kan^R, GFP:ura4⁺, TAP:kan^R, RFP:kan^R, RFP:hyg^R, RFP:ura4⁺, mCherry:kan^R, mTomato:kan^R, or mApple:nat^R cassettes as previously described (Bahler *et al.*, 1998). Standard cloning methods were used to generate the mApple:nat^R cassette from a plasmid generously provided by Dr. Ryoma Ohi (Vanderbilt University, Nashville, TN). A lithium acetate method was used in *S. pombe* tagging transformations (Keeney and Boeke, 1994), and integration of tags

was verified using whole-cell PCR or fluorescence microscopy. Introduction of tagged loci into other strains was accomplished using standard *S. pombe* mating, sporulation, and tetrad dissection techniques. The *nbl1*(1-69) truncation was established in a wild-type diploid strain through the introduction of a stop codon and kan^R cassette after proline 69 via tagging. Expression of *nmt41 nbl1*(1-95) was controlled in *nbl1*-shutoff strains by the addition or removal of 5 µg/ml thiamine to the medium.

For block of *nda3-KM311* strains in prometaphase, cells were grown at 32°C and then shifted to 17°C for 7 h. Subsequent release of these cells to the permissive temperature at 32°C allowed for synchronous progression into anaphase.

For spot assays, cells were grown to midlog phase at 25°C, 10 million cells were resuspended in 1 ml water, and 1 ml serial dilutions were made. 2.5 µL of each dilution were plated on YE, YE plus DMSO alone, or YE plus low dose (0.2 µM) latrunculin A in DMSO. Plates were incubated at 25°C, 27°C, 29°C, or 32°C for 4 to 5 d.

nbl1 Disruption, Shutoff, and Overexpression

Disruption of *nbl1*⁺ was achieved by PCR-based one-step homologous recombination (Bahler *et al.*, 1998). Specifically, the *nbl1*(1-95) fragment was targeted for deletion using *ura4*⁺ as the selectable marker. After transformation of an *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h⁻/h⁺* diploid with the relevant amplified fragment, stable integrants were selected and the *nbl1*(1-95) deletion was confirmed by PCR. Tetrad dissection of this diploid strain (KGY6731) was performed using standard techniques. For spore germination experiments using KGY6731, sporulating diploids were digested with glusulase at 32°C, allowed to recover in YE at 32°C for 1 h, and then grown in EMM plus adenine and leucine overnight at 25°C.

A pZERO-2 vector with *nbl1*(1-95) cDNA was generated by Integrated DNA Technologies. *nbl1*(1-95) cDNA was excised from this vector and cloned into pREP41, placing *nbl1*(1-95) expression under control of pREP41's thiamine-repressible *nmt41* promoter (Basi *et al.*, 1993). The *nmt41-nbl1*(1-95) fragment was subcloned into a pJK148 vector, linearized within the *leu1* gene with NruI, transformed into KGY247, and an integrant at the *leu1* locus was identified (KGY7846). In addition, a pSK vector with *nbl1*(1-95) cDNA and flanking sequences was constructed, and *nbl1*(1-95) cDNA and flanking sequences were excised from this vector and inserted into a pIRT2 vector. KGY6731 was then transformed with this plasmid, and transformed KGY6731 was sporulated in nitrogen-lacking EMM. Spores were subsequently isolated using glusulase, and *nbl1*-disrupted cells covered by the plasmid (KGY7841) were selected on EMM plus adenine. KGY7841 was then crossed to KGY7846, and KGY7919, a *nbl1*-shutoff strain which can grow on EMM plus adenine but not on EMM plus adenine and thiamine, was selected. A pIRTSMART vector with *nbl1*⁺ cDNA was also generated by Integrated DNA Technologies. *nbl1*⁺ cDNA was excised from this vector and cloned into pREP1, placing *nbl1*⁺ expression under control of pREP1's thiamine-repressible promoter (Maundrell, 1990, 1993).

Construction of the *nbl1* Phosphosite Mutant

The *nbl1*-T91A mutation was introduced by site-directed mutagenesis into the pIRT2 vector containing *nbl1*(1-95) cDNA and flanking sequences. KGY6731 was then transformed with this plasmid, and haploid *nbl1*-disrupted cells covered by the plasmid were selected. This strain (KGY8048) was then grown to midlog phase in YE, and 10 million cells were plated on YE plus 5-FOA to select for the appropriate replacement strain, which was confirmed by PCR and sequence analysis.

Protein Methods and Mass Spectrometry

Cells were lysed by bead disruption in NP-40 lysis buffer in either native or denaturing conditions as previously described (Gould *et al.*, 1991), except with the addition of 0.1 to 0.5 mM diisopropyl fluorophosphate (Sigma-Aldrich, St. Louis, MO). Proteins were immunoprecipitated by anti-Myc (9E10), anti-GFP (Roche), or anti-FLAG (M2; Sigma-Aldrich) antibodies. For comparison of the Ark1-GFP levels from different genetic backgrounds, an aliquot of cell lysates was taken to detect Cdc2 levels (using anti-PSTAIR) and protein levels were adjusted before performing immunoprecipitations. Immunoblot analysis was performed as previously described (Wolfe *et al.*, 2006) except that secondary antibodies were conjugated to Alexa Fluor 680 (Invitrogen, Carlsbad, CA) or IRDye800 (LI-COR Biosciences) and visualized using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE). Purification of Clp1-C286S-TAP and Nbl1(1-95)-TAP and subsequent identification of Clp1- and Nbl1-interacting proteins by mass spectrometry were performed as previously described (Gould *et al.*, 2004; Roberts-Galbraith *et al.*, 2009).

In Vitro Kinase Assays

The Ark1 kinase assay was performed as previously described (Petersen *et al.*, 2001; Ohi *et al.*, 2004) with minor modifications. Briefly, anti-GFP immunoprecipitates were washed three times in 1 ml NP40 buffer and three times in 1 ml 1× kinase buffer (20 mM K-HEPES, pH7.8, 5 mM MgCl₂, 1 mM EGTA, and 1 mM DTT). After washes, liquid was aspirated and 4 µL 5× kinase buffer was added to resuspend the beads. 5 µg purified Histone H3.2 (New England

Biolabs), 50 μ M cold ATP, and 5 μ Ci of [γ - ^{32} P] ATP (GE Healthcare, Piscataway, NJ) were added. After incubation with shaking at 30°C for 30 min, reactions were terminated by addition of 5 μ l of 5 $\times$ SDS-PAGE loading buffer, and proteins were resolved on a 4% to 12% Bis-Tris gel (Invitrogen). The gel was cut in half and the upper portion (30 to 90 kDa) was transferred to a PVDF membrane and blotted with anti-GFP antibodies to detect Ark1-GFP. The lower part (10 to 30 kDa) of the same gel was stained by Coomassie blue dye to visualize Histone H3.2, and, after drying of the gel, autoradiography was used to check incorporation of ^{32} P.

For the Cdk1 kinase assay, $\approx$ 50 ng of recombinant Cdk1 kinase complex, purified from baculovirus-infected insect cells as previously described (Yoon *et al.*, 2002), was incubated with 200 ng of bacterially produced His6-Nbl1(1-95) in a reaction buffer containing 50 mM Tris pH 7.4, 10 mM MgCl₂, 2 μ M DTT, 10 μ M unlabeled ATP, and 5 μ Ci of [γ - ^{32} P] ATP. After incubation with shaking at 30°C for 30 min, reactions were terminated with 5 $\times$ sample buffer, and samples were boiled and separated by SDS-PAGE. Coomassie blue staining and autoradiography were subsequently performed.

Microscopy

For live-cell microscopy, wild-type cells, unless otherwise noted, were grown to midlog phase at 25°C and imaged. Temperature-sensitive strains were first grown to midlog phase at 25°C, synchronized in G2 phase using a 7% to 30% lactose gradient, and shifted to 36°C for 2 to 3 h before live-cell microscopy. All live images were acquired using a spinning disk confocal microscope (Ultraview LCI; PerkinElmer, Foster City, CA) equipped with a 100 $\times$ NA 1.40 Plan-Apochromat oil immersion objective, a 488-nm argon ion laser (GFP), and a 594-nm helium neon laser (RFP, mCherry, mTomato, mApple). Images were taken via a charge-coupled device camera (Orca-ER; Hamamatsu Photonics) and subsequently processed using Metamorph 7.1 software (MDS Analytical Technologies, Molecular Devices, Sunnyvale, CA). Z-section slices were 0.5 μ m. Time-lapse images were taken of cells on YE agar pads sealed with Valap (a Vaseline, lanolin, and paraffin mixture). An objective heater system (Biopatch) was used to maintain the restrictive temperature during imaging of temperature-sensitive strains.

To visualize DNA or cell walls, cells were fixed in 70% ethanol and stained with DAPI or methyl blue. Images were acquired using a microscope (Axioskop II; Carl Zeiss, Thornwood, NY) equipped with a 100 $\times$ NA 1.40 Plan-Apochromat oil immersion objective and a halogen lamp. OpenLab 4.0.3 software (PerkinElmer) was used to process these images.

Images of yeast colonies were acquired by focusing a camera (PowerShot SD750; Canon, Tokyo, Japan) through a microscope (Universal; Carl Zeiss) equipped with a 20 $\times$ NA 0.32 objective.

RESULTS

Nbl1 Is an Essential Protein which Associates with and Regulates Activity of the Chromosomal Passenger Complex

To identify Clp1-associated proteins in the fission yeast, *Schizosaccharomyces pombe*, we performed tandem affinity purification (TAP) of the phosphatase-dead Clp1-C286S mutant followed by mass spectrometry. One of the proteins identified through this approach is a sequence orphan, SPBC725.12 (Figure 1A). SPBC725.12 has not been previously characterized except for its identification as an essential “meiotically upregulated gene” (*mug*) (Mata *et al.*, 2002). For simplicity, we will subsequently refer to SPBC725.12 as Nbl1 (novel Borealin-like 1) based on the studies described in this article.

We confirmed that *nbl1* is an essential gene by analyzing tetrads from a diploid strain having one copy of *nbl1* disrupted with *ura4*⁺. Tetrads showed a 2:2 ratio of Ura[−] viable to inviable spores or only one Ura[−] viable spore (Supplementary Figure 1A), indicating that Nbl1 is an essential protein. To examine the cause of inviability when *nbl1* is disrupted, we sporulated diploids heterozygous for the *nbl1* disruption and allowed only Ura⁺ spores, containing the *nbl1* disruption, to germinate. DAPI staining indicated that 74% (148/200) of septated *nbl1*-disrupted cells exhibited a cut phenotype, with the septum slicing through the DNA (Figure 1B). Cut phenotypes arise from defects in sister chromatid separation (Yanagida, 1998) and were not observed in wild-type cells (Supplementary Figure 1B). Thus, Nbl1 appears to affect chromosome segregation.

Figure 1. The Clp1-associated protein, Nbl1, is essential for chromosome segregation. (A) Analysis of mass spectrometry data after TAP of *clp1*-C286S-TAP (KGY5423), with percent coverage, total peptide number, and unique peptide number for proteins of interest presented. (B) A diploid heterozygous for the *nbl1* disruption (KGY6731) was sporulated, and spores having the *nbl1* disruption were selected using the *ura4*⁺ marker. Cells were fixed in ethanol and stained with DAPI to visualize DNA. Cells are outlined in white, septa are represented by dashed white lines, and cut phenotypes are indicated by white arrows. (C) *nbl1*-shutoff cells (KGY7919) were grown in the presence of thiamine for 10 h, fixed with ethanol, and stained with DAPI to visualize DNA. Cells are outlined in white, and septa are indicated by dashed white lines. (D) pREP1-*nbl1*⁺ *ark1*-GFP cells (KGY8663) were grown in the presence of thiamine, transferred to medium lacking thiamine for 24 h, fixed in ethanol, and stained with DAPI and methyl blue to visualize DNA and cell walls, respectively. Bars, 5 μ m.

To verify that the observed cut phenotypes are in fact due to *nbl1* malfunction, we rescued the null mutation with a genomic clone containing *nbl1*⁺ cDNA (data not shown) and also developed a *nbl1*-shutoff strain. The original open reading frame predicted for Nbl1 in the Pombe Genome Database was a truncated version, comprising only the first 95 amino acids of Nbl1's 141 amino acids (Supplementary Figure 1C). Thus, we initially placed *nbl1*(1-95) cDNA under control of the thiamine repressible *mtt41* promoter (Basi *et al.*, 1993) and integrated this fragment at the *leu1* locus in a *nbl1*-disrupted strain. This truncated cDNA was sufficient to rescue the *nbl1* disruption, indicating that the C terminus of Nbl1 is dispensable for proper Nbl1 function. Consistent with this, most sequence conservation between *S. pombe* Nbl1 and homologues in *S. octosporus* and *S. japonicus* lies in the N terminus (Supplementary Figure 1C). Because Nbl1(1-95) was fully functional, we used this truncation interchangeably with full-length Nbl1 in our studies. Shutoff of *nbl1*(1-95) expression produced cut phenotypes (Figure 1C) similar to those which were seen in *nbl1*-disrupted cells (Figure 1B). Therefore, the previously described defects in chromosome segregation are attributable to disruption of *nbl1*.

To examine whether *nbl1* overexpression is tolerated by cells, *nbl1*⁺ cDNA was placed under control of the *mtt1* promoter in pREP1 (Maundrell, 1990, 1993) and transformed into wild-type cells. On removal of thiamine, cells overexpressing *nbl1* failed to form colonies (Supplementary Figure 1D) and often showed unequal segregation of DNA (Figure 1D). Thus, not only is *nbl1* an essential gene, but its protein levels must be regulated properly to ensure appropriate chromosome segregation.

Figure 2. Nbl1 associates with the CPC and affects Ark1 function and stability. (A) Analysis of mass spectrometry data after TAP of *nbl1(1-95)*-TAP (KGY6825), with percent coverage, total peptide number, and unique peptide number for proteins of interest presented. (B) Anti-GFP immunoprecipitates from the indicated strains were blotted with anti-FLAG and anti-GFP antibodies. (C) Ark1-GFP was immunoprecipitated from the indicated strains and incubated with histone H3 in the presence of γ - ^{32} P-ATP. Where appropriate, shutoff of *nbl1*-shutoff *ark1*-GFP (KGY8275) was achieved before cell lysis via addition of thiamine (T) for 10 h. Kinase reactions using Ark1-GFP immunoprecipitated from *ark1*-GFP cells (KGY4702) were carried out in either the presence or absence of the Aurora B inhibitor, ZM447439. The amount of histone H3 in the reactions was determined by Coomassie blue staining, and the amount of Ark1-GFP in the reactions was determined by immunoblotting anti-GFP immunoprecipitates with an anti-GFP antibody. An untagged strain (KGY246) was used as a negative control in the anti-GFP immunoprecipitation. (D) Anti-GFP immunoprecipitates were blotted with an anti-GFP antibody. Ark1-GFP levels were normalized to Cdc2, which was quantified by blotting cell lysates with an anti-PSTAIR antibody. Shutoff of *nbl1*-shutoff *ark1*-GFP (KGY8275) was achieved before cell lysis via addition of thiamine for 10 h.

To identify proteins with which Nbl1 interacts and functions, we performed a TAP of Nbl1(1-95) followed by mass spectrometry. Interestingly, Bir1/Cut17, the *S. pombe* Survivin homologue, and Pic1, the *S. pombe* INCENP homologue, which both had been previously identified along with Nbl1 in the Clp1-C286S-TAP (Figure 1A), were identified along with Clp1 in a Nbl1(1-95)-TAP (Figure 2A). We confirmed the association between Nbl1(1-95) and Bir1 by traditional coimmunoprecipitation (Figure 2B). In addition to Bir1 and Pic1, Ark1, which is the *S. pombe* Aurora B homologue, is a known member of the fission yeast chromosomal passenger complex (CPC) (Ruchaud *et al.*, 2007). We did not detect Ark1 in the Nbl1(1-95)-TAP. However, TAPs of the Survivin homologue in *S. cerevisiae* also failed to recover the Aurora B homologue (Sandall *et al.*, 2006; Widlund *et al.*, 2006), suggesting that the kinase does not remain bound to its nonenzymatic subunits during a standard TAP procedure. Consistent with physical associations between Nbl1 and known CPC components, we also noted a variety of negative genetic interactions among tagged alleles of CPC components and *nbl1(1-95)*. Specifically, *nbl1(1-95)*-TAP

bir1-FLAG, *nbl1(1-95)*-TAP *bir1*-GFP, *nbl1(1-95)*-TAP *pic1*-myc, and *nbl1(1-95)*-FLAG *bir1*-TAP were all synthetically lethal (data not shown).

Given the apparent association of Nbl1 with known CPC subunits, we tested the ability of Ark1, immunoprecipitated from wild-type and *nbl1*-shutoff strains, to phosphorylate histone H3 in vitro. Ark1-mediated phosphorylation of histone H3 is critical to establishment of condensed chromatin (Petersen *et al.*, 2001; Petersen and Hagan, 2003), and thus histone H3 phosphorylation can be used as a readout for CPC activity. Not surprisingly, histone H3 was phosphorylated by Ark1-GFP from wild-type cells (Figure 2C, lane 3), and such phosphorylation was decreased in the presence of an Aurora B kinase inhibitor, ZM447439 (Figure 2C, lane 2). The specificity of this kinase assay for Ark1 was confirmed using higher concentrations of the Aurora B inhibitor (Supplementary Figure 2). Interestingly, phosphorylation of histone H3 was nearly abolished when Ark1 was immunoprecipitated from cells in which *nbl1* expression had been repressed (Figure 2C, lane 4). Although Nbl1 was required for the stability of Ark1 (Figure 2D) in addition to the stability of Bir1 and Pic1 (data not shown), we performed the kinase assay after Ark1 levels had been adjusted for equivalence (Figure 2C, bottom panel). Thus, these results indicate that Nbl1 not only associates with CPC components but is required for proper activity and stability of the CPC.

Nbl1 and the CPC Are Interdependent for Proper Localization

Because Nbl1 and known CPC subunits exhibit physical and functional associations, we next examined whether Nbl1 localizes similarly to chromosomal passenger proteins. As shown by time-lapse microscopy of Nbl1(1-95)-GFP and Sid4-RFP, a marker for the *S. pombe* spindle pole body (Chang and Gould, 2000), Nbl1(1-95)-GFP localized to dots consistent with centromeres in metaphase and relocated to the mitotic spindle and the spindle midzone during anaphase (Figure 3A and Supplementary Movie 1). This behavior was similar to that of Ark1, Pic1, and Bir1 (Supplementary Figure 3A; Morishita *et al.*, 2001; Petersen *et al.*, 2001; Rajagopalan and Balasubramanian, 2002; Huang *et al.*, 2005). In addition, Nbl1-GFP localized identically to the Nbl1(1-95)-GFP truncation (Supplementary Figure 3B), again demonstrating that Nbl1(1-95) is sufficient for proper localization and function of Nbl1.

During interphase and upon septation, Nbl1-GFP (Supplementary Figure 3B) and Nbl1(1-95)-GFP (Figure 3A and Supplementary Movie 1) accumulated within a distinct compartment of the nucleus. Previous studies have shown that the *S. pombe* CPC is nucleolar in interphase (Vanoosthuyse *et al.*, 2007). Colocalization of Nbl1(1-95)-GFP and Nog1-RFP, a nucleolus marker (Matsuyama *et al.*, 2006), confirmed that Nbl1 is also nucleolar in interphase (Figure 3B). Additionally, imaging of Nbl1-GFP with Nuf2-RFP, a kinetochore marker (Nabetani *et al.*, 2001), verified that the Nbl1-GFP dots seen during metaphase were consistent with Nbl1 localization to centromeres in metaphase (Supplementary Figure 3C). Though Nbl1-GFP and Nuf2-RFP did not completely overlap, their side-by-side orientation is similar to that which has previously been observed for other centromeric CPC proteins and kinetochore markers in metaphase (Vanoosthuyse *et al.*, 2007). To further validate that Nbl1 colocalizes with the CPC during mitosis, we imaged Nbl1(1-95)-GFP along with Pic1-mCherry. Nbl1(1-95)-GFP and Pic1-mCherry colocalized to centromeres in metaphase and to the mitotic spindle and the spindle midzone in anaphase (Figure 3C). Therefore, taken together with our evidence for a phys-

Figure 3. Nbl1 colocalizes with CPC subunits in dependent fashions. I indicates interphase; M, metaphase; A, anaphase; S, septation. (A) Representative live-cell GFP and RFP images were acquired for *nbl1(1-95)-GFP sid4-RFP* cells (KGY7563) at various stages of the cell cycle, and the GFP and RFP images were merged. The septum is indicated by a dashed white line. (B) Representative live-cell bright field (BF), GFP, and RFP images were acquired for a *nbl1(1-95)-GFP nog1-RFP* cell (KGY8267) in interphase, and the GFP and RFP images were merged. (C) Representative live-cell BF, GFP, and mCherry images were acquired for *nbl1(1-95)-GFP pic1-mCherry* cells (KGY7796) in metaphase and anaphase, and GFP and mCherry images were merged. (D through F) Representative live-cell GFP and RFP or mApple images were acquired for *cut17-275 nbl1-GFP sid4-RFP* (KGY8633) (D), *pic1-T269 nbl1-GFP sid4-RFP* (KGY8625) (E), and *ark1-T7 nbl1-GFP sid4-mApple* cells (KGY8629) (F) progressing through mitosis after G2 synchronization and shift to 36°C, and the GFP and RFP or mApple images were merged. Septa are indicated by dashed white lines. (G through I) Representative live-cell GFP and RFP images were acquired for *nbl1-shutoff bir1-GFP sid4-RFP* (KGY8290), *nbl1-shutoff pic1-GFP sid4-RFP* (KGY8493), and *nbl1-shutoff ark1-GFP sid4-RFP* cells (KGY8771) after 10 h of *nbl1* repression with thiamine (+T), and GFP and RFP images were merged. Septa are indicated by dashed white lines. Bars, 5 μ m.

ical association between Nbl1 and the CPC, it seems likely that Nbl1 travels as a part of the CPC.

We next examined whether Nbl1 requires Bir1, Pic1, or Ark1 for proper mitotic localization. Nbl1-GFP localized normally in wild-type cells at 36°C (Supplementary Figure 3D). At this restrictive temperature in *cut17-275* and *pic1-T269* temperature-sensitive cells, Nbl1-GFP accumulated at centromeres as usual (16 of 16 cells, 100%; and 38 of 38 cells, 100%, respectively) but commonly failed to localize to the mitotic spindle or the spindle midzone correctly. Instead, Nbl1-GFP often localized diffusely in the nucleoplasm in *cut17-275* and *pic1-T269* cells (57 of 72 cells, 79%; and 58 of 88 cells, 66%, respectively; Figure 3, D and E), indicating that Bir1 and Pic1 are necessary for its proper spindle localization. Additionally, Nbl1-GFP segregated unevenly at the completion of anaphase (Figure 3, D and E), consistent with unequal segregation of DNA in CPC mutants (Samejima *et al.*, 1993; Levenson *et al.*, 2002). At the restrictive temperature in *ark1-T7* temperature-sensitive cells, however, Nbl1-GFP localized normally to both centromeres (53 of 53 cells, 100%) and the mitotic spindle (71 of 74 cells, 96%; Figure 3F). Yet, similar to Nbl1-GFP in *cut17-275* and *pic1-T269* septated cells, Nbl1-GFP segregated unevenly (Figure 3F). In sum, although Nbl1-GFP localized independently of other CPC components to centromeres, it required the function of Bir1 and Pic1 for proper spindle localization.

Next, we investigated whether localization of known *S. pombe* CPC subunits requires Nbl1. As previously demonstrated (Morishita *et al.*, 2001; Petersen *et al.*, 2001; Rajagopalan and Balasubramanian, 2002; Huang *et al.*, 2005), Bir1-GFP, Pic1-GFP, and Ark1-GFP localized to centromeres in metaphase and to the mitotic spindle and the spindle midzone in anaphase of wild-type cells (Supplementary Figure 3A). This localization was maintained in *nbl1*-shutoff cells in the absence of thiamine (Supplementary Figure 3, E–G). However, after repression with thiamine, Bir1-GFP, Pic1-GFP, and Ark1-GFP for the most part did not accumulate at centromeres in metaphase cells (Figure 3, G–I), with 11 of 12 (92%), 15 of 20 (75%), and 19 of 21 (90%) cells lacking clear Bir1-GFP, Pic1-GFP, and Ark1-GFP signals, respectively, at the centromeres. Additionally, during anaphase in *nbl1*-repressed cells, Bir1-GFP localized diffusely within the nucleoplasm instead of tightly on the spindle in 31 of 35 (89%) cells (Figure 3G). In contrast, Pic1-GFP localized normally on the mitotic spindle and on the spindle midzone in 32 of 36 (89%) cells (Figure 3H). We nonetheless observed unequal segregation after anaphase of both Bir1-GFP and Pic1-GFP (Figure 3, G and H). Because of the high background signal for Ark1-GFP, it was more difficult to ascertain the localization of Ark1-GFP in anaphase upon repression of *nbl1* expression. However, we observed separated spindle pole bodies in 52 of 72 (72%) anaphase cells in the absence of any detectable Ark1-GFP signal on the spindle (Figure 3I). This observation suggests that Ark1-GFP localization to the spindle and the midzone, similar to that of Bir1-GFP, was impaired by *nbl1* disruption.

Nbl1 Sequence Analysis Reveals Similarities to Human Borealin

Given that Nbl1 associates with known CPC components and colocalizes with them in a dependent manner, it seemed reasonable that Nbl1 might be a fourth subunit of the *S. pombe* CPC, related to Borealin. To pursue this possibility, we analyzed the predicted secondary structure of Nbl1 using the Jpred3 server (Cole *et al.*, 2008). The N-terminal region of Nbl1, from residues 14 to 85, is predicted to be highly α -helical (Figure 4A). Additionally, Nbl1 exhibits

coiled-coil oligomer potential from residues 22 to 56 of this helical region. Using BLAST to search for related proteins, we identified the helical region of Borealin as a sequence homologue. The central helical region of Nbl1, spanning residues 32 to 79, is 20% identical and 52% homologous to residues 25 to 72 of the N terminus of Borealin (Figure 4B). We independently analyzed Nbl1 using the Phyre protein threading server to search for homologues with known structure (Bennett-Lovsey *et al.*, 2008). The first match was the N terminus of human Borealin, with an E value of 0.35 and estimated precision of 85% (PDB = 2RAX, Bourhis *et al.*, 2007). All other matches were unrelated helical proteins with significantly lower similarity (E value >2.7, precision <55%).

The N-terminal region of Borealin encodes an extended helix which interacts with both Survivin and INCENP to form a three-helix bundle (Figure 4C; Jeyaprakash *et al.*, 2007). The Phyre-based model of Nbl1 (N29–H83) is consistent with core features of Borealin important for this interaction, containing both an extended coiled-coil followed by a helical region similar to the region of Borealin which binds to the dimerization arm of Survivin (Figure 4C). Additionally, the specific residues conserved between Borealin and Nbl1 strongly cluster to those residues at the protein-protein interaction surface. Twenty-two of the 25 conserved residues are predicted to be localized to the protein-protein interaction face of Nbl1 (Figure 4D). The three residues positioned on the opposite helical face (E33, R44, R70) are charged residues. One of the strictly conserved residues is proline 69 (Figure 4, B and D). This proline serves to break the long helix of Borealin and positions the helical segments which bind to the dimerization arm of Survivin (Figure 4D). This interaction leads to the observed dimer to monomer transition of Survivin which is important for Survivin function (Bourhis *et al.*, 2007). Interestingly, although cells were viable when we truncated Nbl1 to its first 95 amino acids, truncation of Nbl1 after the conserved proline at residue 69 rendered cells inviable. Specifically, *nbl1*(1–69) cells cut in their first division (Figure 4E). Thus, the additional short helical stretch following the conserved proline in Nbl1 appears to be likewise relevant for CPC function in *S. pombe*. Accordingly, Nbl1 and Borealin share a conserved N-terminal half that is both physically and functionally important. Together with the physical and functional associations of Nbl1 with the CPC, these similarities further support the identification of Nbl1 as the *S. pombe* Borealin homologue.

Clp1 and the CPC Are also Interdependent for Proper Localization

We next analyzed in more detail the association between CPC components and the Clp1/Cdc14 phosphatase, given that they had purified in our Clp1-C286S-TAP. Phosphatase-dead Clp1-C286S-GFP coimmunoprecipitated Nbl1(1–95)-FLAG during and after release from an *nda3-KM311* prometaphase arrest (Figure 5A), suggesting that Clp1 and Nbl1 associate in both metaphase and anaphase. Consistent with this, Clp1-GFP and Nbl1(1–95)-mTomato colocalized to centromeres in metaphase and to the mitotic spindle and the spindle midzone in anaphase (Figure 5B).

Because Clp1 reverses Cdk1-mediated phosphorylation events, we examined whether Nbl1 might be a Cdk1 target that is regulated by its phosphorylation state. Cdk1 can in fact phosphorylate Nbl1(1–95) in vitro (Supplementary Figure 4A), and T91 is the only S/T site matching a Cdk1 consensus within the protein. Replacing T91 with an alanine at the *nbl1* locus revealed that Nbl1 localization was unaffected by this mutation (Supplementary Figure 4B). Analysis

Figure 5. Nbl1 associates with Clp1 and is affected by *clp1* deletion. (A) Indicated strains were lysed either during an *nda3*-KM311 prometaphase block or 30 min after release from this block. Anti-GFP and anti-FLAG immunoprecipitates were blotted with anti-GFP and anti-FLAG antibodies. (B) Representative live-cell GFP and mTomato images were acquired for *clp1*-GFP *nbl1(1-95)*-mTomato cells (KGY7840) in metaphase and anaphase, and GFP and mTomato images were merged. M indicates metaphase; A, anaphase. (C) Representative live-cell GFP and RFP images were acquired for *nbl1*-GFP *sid4*-RFP (KGY8602) and *nbl1*-GFP *sid4*-RFP *clp1*Δ cells (KGY8731) in late anaphase, and GFP and RFP images were merged. (D) Representative live-cell GFP images were acquired for *ase1*-GFP (KGY5488) and *ase1*-GFP *clp1*Δ cells (KGY6142) in late anaphase. Ase1 localizes to the spindle pole bodies in addition to the spindle midzone (Yamashita *et al.*, 2005), and thus the two most outlying dots in each image are indicative of spindle pole body localization. Bars, 5 μm.

($n \geq 20$; Supplementary Figure 5B), Nbl1-GFP did not localize to the midzone correctly in *clp1*Δ cells (Figure 5C). Instead, it tailed off to one pole during anaphase (Figure 5C). We noted that Nbl1-GFP localized in an identical manner in a strain with the phosphatase-dead *clp1*-C286S mutation (Supplementary Figure 6, A and B), additionally supporting the conclusion that Clp1 phosphatase activity is relevant to only the midzone localization of the CPC.

Midzone localization of Ark1 in *S. pombe* has been linked previously to a requirement for Ase1, a microtubule-bundling protein that is necessary for midzone stability (Yamashita *et al.*, 2005). In *clp1*Δ (Figure 5D) and *clp1*-C286S (Supplementary Figure 6C) cells, we observed that Ase1-GFP also tailed off to one pole during anaphase. This suggests that the midzone itself was improperly formed in *clp1*Δ and *clp1*-C286S strains. Thus, mislocalization of Nbl1 and

Figure 6. Clp1 fails to accumulate at the CR in the absence of CPC activity. (A) Representative live-cell bright field (BF), GFP, and RFP images were acquired for *nbl1*-shutoff *clp1*-GFP *sid4*-RFP cells (KGY8730) after 10 h of *nbl1* repression with thiamine (+T), and GFP and RFP images were merged. (B–D) Representative live-cell BF, GFP, and RFP images were acquired for *cut17-275* *clp1*-GFP *sid4*-RFP (KGY8720), *pic1*-T269 *clp1*-GFP *sid4*-RFP (KGY8718), and *ark1*-T7 *clp1*-GFP *sid4*-RFP cells (KGY8721) progressing through mitosis after G2 synchronization and shift to 36°C, and the GFP and RFP images were merged. Clp1 localization at the CR is indicated by white arrows. (E) Representative time-lapse GFP and RFP images were acquired for *ark1*-T7 *clp1*-GFP *sid4*-RFP (KGY8721) and *clp1*-GFP *sid4*-RFP cells (KGY8019) progressing through mitosis after G2 synchronization and shift to 36°C, and GFP and RFP images were merged. Initial images were taken at the metaphase-to-anaphase transition, and subsequent images were taken every two minutes. Bars, 5 μ m.

other CPC components in *clp1* Δ and *clp1*-C286S strains appears to be attributable to faulty midzone formation in the absence of Clp1 phosphatase activity.

We next tested whether disruption of *nbl1* affects Clp1 localization by monitoring Clp1-GFP and Sid4-RFP after repression of *nbl1* expression. In anaphase of wild-type cells, Clp1-GFP localized to both the spindle and the CR (Figure 6E, left column). However, in *nbl1*-shutoff cells, Clp1-GFP localized to the spindle but not the CR during anaphase in 23 of 33 (70%) cells (Figure 6A). In addition, Clp1-GFP often accumulated between separated spindle pole bodies in an unsegregated mass (Figure 6A), consistent with the chromosome segregation defects seen in *nbl1*-disrupted cells. We then examined Clp1-GFP and Sid4-RFP in the CPC temperature strains *cut17-275*, *pic1*-T269, and *ark1*-T7. Though some *cut17-275*, *pic1*-T269, and *ark1*-T7 cells showed localization of Clp1-GFP to the CR in early anaphase at the restrictive temperature (panel I, Figure 6, B–D), most anaphase *cut17-275* (27 of 35, 77%), *pic1*-T269 (24 of 31, 67%), and *ark1*-T7 (36 of 42, 86%) cells lacked any detectable Clp1-GFP signal at the CR (panels III–V, Figure 6, B–D). Instead, Clp1-GFP solely localized to the mitotic spindle and to an unsegregated mass between spindle pole bodies in these cells (panels II–V, Figure 6, B–D). Thus, Clp1-GFP accumulation at the CR was abnormal in all CPC mutations tested.

To study the defects of Clp1 localization in more detail, Clp1-GFP and Sid4-RFP were imaged by time-lapse microscopy in the *ark1*-T7 strain. As suggested by the still images, Clp1-GFP never accumulated properly on the CR during anaphase. In some cases, Clp1 failed to localize to any degree on the CR (Figure 6E, middle column). In other cases, we detected faint CR localization of Clp1-GFP, but this signal dissipated prematurely and never reached its normal intensity (Figure 6E, right column). These observations are in striking contrast to what was seen in wild-type *clp1*-GFP *sid4*-RFP cells, in which Clp1-GFP localized strongly to the

CR initially in metaphase (Figure 6E, left column, t = 0) and continued on the CR through anaphase (Figure 6E, left column, t = 2–10). Accordingly, disruption of CPC function severely affected Clp1 accumulation at the CR.

The CPC Genetically Interacts with the *S. pombe* Cytokinetic Apparatus

Clp1 accumulation at the CR is required for the fidelity of cytokinesis (Trautmann and McCollum, 2005; Chen *et al.*, 2008; Clifford *et al.*, 2008). Furthermore, temperature-sensitive alleles of CR components exhibit a strong genetic interaction with *mid1* $\Delta_{431-481}$, which encodes a Mid1 mutant that lacks the region necessary to recruit Clp1 to the CR (Clifford *et al.*, 2008). Therefore, given the relevance of the CPC to Clp1 accumulation at the CR, we used a genetic approach to examine whether previously unrecognized connections between the *S. pombe* CPC and the cytokinetic apparatus exist. Interestingly, we observed negative genetic interactions (Figure 7A) between *ark1*-T7 and temperature-sensitive alleles of the following genes: *cdc12*, which encodes a critical cytokinetic formin (Pelham and Chang, 2002; Kovar *et al.*, 2003); *rng2*, which encodes an IQGAP-related protein required for CR formation (Eng *et al.*, 1998); and *sid2*, which encodes a kinase that functions in septation initiation (Balasubramanian *et al.*, 1998). The fact that the *ark1*-T7 *cdc12*-112 double mutant failed to grow as well as either single mutant at the permissive temperature was consistent with the observation that some of these double mutants failed in their first division on tetrad plates (Supplementary Figure 7A). Furthermore, the observed negative genetic interactions between *ark1*-T7 and CR mutant alleles specifically correlated with an exacerbation of cytokinesis and septation defects, for double mutants showed a greater proportion of defective cells than either of the relevant single mutants (Figure 7, B and C). In addition, we noted that two temperature-sensitive alleles of *bir1*, *cut17-275* and *bir1*-T1,

Figure 7. *ark1-T7* exhibits negative genetic interactions with CR mutant alleles. (A) Serial 10-fold dilutions of cells of the indicated genotype were spotted on YE plates and incubated at 25°C, 27°C, or 29°C for 4 to 5 d. (B) The indicated strains were grown to midlog phase at 25°C and then shifted to 29°C for 10 h. Cultures were kept in log phase by the addition of prewarmed media. Cells were ethanol fixed and stained with DAPI and methyl blue to visualize DNA and cell walls, respectively. Bar, 5 μ m. (C) >300 cells for each strain imaged in B were counted and grouped into one of the indicated categories.

also showed a negative genetic interaction with *cdc12-112* (Supplementary Figure 7, B and C) and that the relevant double mutants likewise exhibited an increased instance of cytokinesis and septation defects (Supplementary Figure 7, D and E). We did not, however, detect genetic interactions between CPC temperature-sensitive alleles and the *mid1 Δ ₄₃₁₋₄₈₁* mutant allele (Supplementary Figure 8), consistent with the primary role of the CPC in cytokinesis being to mediate Clp1 accumulation at the CR.

We next examined whether *ark1-T7* cells are sensitive to latrunculin A (Lat A), a drug which inhibits actin polymerization (Ayscough *et al.*, 1997). Though high-dose Lat A treatment (>10 μ M) results in a complete loss of F-actin structures in wild-type cells (Pelham and Chang, 2001), low-dose Lat A treatment (0.2 μ M) is not lethal (Mishra *et al.*, 2004). However, low-dose Lat A treatment mildly perturbs the cytokinetic machinery, and cells defective in cytokinesis, such as those lacking *clp1*, are sensitive to these low doses (Figure 8, A–C) (Mishra *et al.*, 2004). Though growth of

ark1-T7 on control and low-dose Lat A-containing plates was similar at 25°C, growth of *ark1-T7* was slightly impaired at 27°C and considerably impaired at 29°C on low-dose Lat A-containing plates (Figure 8A). Consistent with this observation, *ark1-T7* cells showed significant cytokinesis and septation defects in the presence of low-dose Lat A in liquid culture (Figure 8, B and C). Therefore, *ark1-T7* cells are sensitive to mild inhibition of actin polymerization at semi-permissive temperature. These data support the notion that the fission yeast CPC promotes the process of cytokinesis.

DISCUSSION

Similar to their orthologues in other organisms (Vagnarelli and Earnshaw, 2004; Vader *et al.*, 2006; Ruchaud *et al.*, 2007), the *S. pombe* proteins Ark1, Pic1, and Bir1 function within a chromosomal passenger complex that regulates critical mitotic events such as chromosome segregation and spindle elongation (Morishita *et al.*, 2001; Petersen *et al.*, 2001; Leverson

Figure 8. *ark1-T7* cells are sensitive to low-dose Lat A. (A) Serial 10-fold dilutions of cells of the indicated genotype were spotted on YE plates containing DMSO alone (control) or DMSO plus low-dose Lat A and incubated at 25°C, 27°C, or 29°C for 4 d. (B) The indicated strains were grown to midlog phase at 25°C, and then DMSO or DMSO containing low-dose Lat A was added. Cultures were immediately shifted to 29°C for 10 h. Cultures were kept in log phase by the addition of prewarmed media. Cells were ethanol-fixed and stained with DAPI and methyl blue to visualize DNA and cell walls, respectively. Bar, 5 μ m. (C) >300 cells for each strain imaged in B were counted and grouped into one of the indicated categories.

et al., 2002; Rajagopalan and Balasubramanian, 2002; Huang *et al.*, 2005; Widlund *et al.*, 2006). However, whether these three proteins operate alone in this complex has remained unclear, for a Borealin homologue has not been previously described in *S. pombe*. Here, we characterize Nbl1 as an *S. pombe* Borealin-like protein based on its association with known CPC components, its colocalization with other CPC subunits during mitosis, and its sequence and proposed structural similarity to human Borealin. Consistent with previous data directly linking Borealin to Aurora B activation (Jelluma *et al.*, 2008), we additionally demonstrate that Nbl1 is required for Ark1 activity. Furthermore, we show that the *S. pombe* CPC influences the process of cytokinesis, at least partially by controlling the localization of the fission yeast Cdc14-family phosphatase, Clp1.

Identification of Borealin homologues in yeasts has previously been hampered by the fact that they do not possess open reading frames with significant primary sequence identity to human Borealin. Even the Nbl1(1-95) sequence, which was sufficient to rescue disruption of *nbl1* and to support proper localization and associations of the CPC during mitosis, possesses only a few residues which are identical to residues at the same positions in Borealin. While this manuscript was in preparation, a study was published describing a budding yeast Borealin homologue (Nakajima *et al.*, 2009). In this article, the authors also identified putative Borealin homologues in almost 200 species through Hidden Markov Model-based searches. One of the putative homologues which they noted in this analysis (Nakajima *et al.*, 2009) is the *S. pombe* protein, which we have described in this study. Thus, our work supports their findings and confirms that Nbl1 is in fact an *S. pombe* Borealin homologue.

We fortuitously discovered that a truncation of Nbl1 lacking the last 46 amino acids localizes correctly, interacts with other CPC components, and does not exhibit genetic inter-

actions consistent with a loss of function. Though a recent study suggested that full-length Borealin is required for the formation of a competent Borealin-Survivin complex which can bind INCENP (Zhou *et al.*, 2009), it has been posited that Borealin-like proteins in lower eukaryotes retain only structures involved in formation of the critical three-helix bundle of Borealin-Survivin-INCENP homologues (Nakajima *et al.*, 2009). Proper function of Nbl1(1-95) supports this hypothesis because it maintains the core three-helix bundle region. Nbl1(1-95) also retains a second helical stretch that follows a conserved proline. Given that this helical stretch is additionally necessary for viability, this region represents another critical structural domain. It will be interesting to analyze whether this region, similar to the helical stretch following the conserved kink in human Borealin (Bourhis *et al.*, 2007), blocks homodimerization of the Survivin homologue.

A significant question in the CPC field concerns how CPC localization is regulated by its different subunits. In human cells, CPC proteins are strikingly interdependent, with knockdown or disruption of any of the CPC subunits impeding proper localization of the others (Honda *et al.*, 2003; Gassmann *et al.*, 2004). Although mutation of Bir1 has been found to impede localization of Ark1 and Pic1 (Morishita *et al.*, 2001; Rajagopalan and Balasubramanian, 2002; Huang *et al.*, 2005), a comprehensive dissection of CPC interdependency in localization has been lacking in *S. pombe*. Here, we undertook a thorough analysis of Nbl1-CPC interdependency and noted additional associations relevant to CPC localization. An intriguing possibility suggested by our data is that Nbl1 mediates centromeric targeting of the CPC. Nbl1 localization to centromeres is independent of Bir1, Pic1, and Ark1, whereas centromere localization of Bir1, Pic1, and Ark1 requires Nbl1. Additionally, similar to human Borealin (Klein *et al.*, 2006), Nbl1 binds DNA (unpublished data). Future studies in the genetically tractable *S. pombe* could

help clarify the contribution of Borealin homologues to CPC centromere targeting.

Our data furthermore highlight that Clp1, similar to Cdc14 in *S. cerevisiae* (Pereira and Schiebel, 2003; Stoepel *et al.*, 2005), is a bonafide CPC-interacting protein. Ark1 has previously been shown to associate with Clp1 during mitosis (Trautmann *et al.*, 2004), and we additionally identified Bir1, Pic1, and Nbl1 in our Clp1-C286S-TAP complexes. The fact that Clp1 and the CPC colocalize during mitosis furthermore confirms their close association. An obvious question stemming from these observations is whether *S. pombe* CPC proteins are directly regulated by their phosphorylation state. While an in-depth analysis of CPC phosphoregulation in *S. pombe* is beyond the scope of this study, we have found that Nbl1, similar to human Borealin (Kaur *et al.*, 2007), can be phosphorylated by Cdk1 in vitro and that there are numerous sites of phosphorylation matching the Cdk1 consensus on Bir1 and Pic1. Similar to the recent observation that Survivin function in chicken cells does not require Cdk phosphorylation of a known phosphosite (Yue *et al.*, 2008), mutation of the sole Cdk1 consensus site on Nbl1 did not apparently affect its function or localization. However, there might be a combinatorial effect of CPC subunit phosphorylation, similar to the situation in *S. cerevisiae* in which redundant Cdk1-mediated phosphorylation blocks DNA rereplication (Nguyen *et al.*, 2001). Thus, an overt phenotype might only be revealed by eliminating most or all Cdk1 phosphorylation of the CPC.

Currently, it is also unclear whether dephosphorylation of *S. pombe* CPC proteins by Clp1 occurs and, if so, whether this is important for CPC function. The fact that Nbl1, Bir1, and Pic1 copurified with the substrate-trapping Clp1-C286S mutant suggests, however, that at least one of the three is a direct target of Clp1. This possibility is supported by a previous report indicating that Clp1 phosphatase activity is required for Clp1 to affect chromosome segregation through the CPC (Trautmann *et al.*, 2004). Although this issue is unresolved, a few points regarding CPC localization in *clp1Δ* cells deserve noting. Though it was suggested that deletion of *clp1* affects localization of CPC subunits to centromeres (Trautmann *et al.*, 2004), our data indicate that the CPC localizes normally to centromeres in the absence of Clp1. Also, unlike Cdc14 (Pereira and Schiebel, 2003), Clp1 is not required for the initial spindle recruitment of any CPC component in *S. pombe*. Therefore, it is most likely that if Clp1 affects the CPC directly, it would influence its localization dynamics or its specific activity. Although Nbl1 localization to the midzone is disrupted in both *clp1Δ* and *clp1-C286S* cells, we have found that the midzone itself is disrupted in the absence of Clp1 activity. In *S. cerevisiae*, Cdc14 controls midzone formation via de-phosphorylation of Ase1 (Khmelniskii *et al.*, 2007). Our data suggest that Clp1-Ase1 interactions might similarly control midzone assembly in *S. pombe*.

It had not been examined previously whether the CPC in any organism affects Cdc14 family function. We found, unexpectedly, that Clp1 accumulation at the CR was defective in all tested CPC mutations. Clp1 contributes to CR stability and the fidelity of cytokinesis (Trautmann and McCollum, 2005; Chen *et al.*, 2008; Clifford *et al.*, 2008). Yet, because Clp1 is nonessential in *S. pombe*, its loss at the CR perturbs, but does not normally prevent, cytokinesis. Consistent with the CPC affecting this aspect of cytokinesis integrity, *ark1-T7* cells are sensitive to low-dose Lat A treatment and temperature-sensitive alleles of genes encoding CPC and CR components display negative genetic interactions. Therefore, although Ark1 is not essential for *S. pombe* cytokinesis (Pe-

tersen and Hagan, 2003), our data indicate that Ark1 does play a role in this process.

The CPC has similarly been implicated in *S. cerevisiae* cytokinesis. In *S. cerevisiae*, distinct passenger complexes control septin organization in anaphase (Gillis *et al.*, 2005; Thomas and Kaplan, 2007), and the Aurora B homologue mediates a NoCut cytokinesis checkpoint by recruiting abscission inhibitors when DNA fails to segregate out of the cleavage plane (Norden *et al.*, 2006; Mendoza *et al.*, 2009). Though the extent of the contribution of *S. pombe* CPC to cytokinesis is currently unclear, it is unlikely to monitor DNA remaining in the division plane. Cut mutations resulting from inhibition of a variety of mitotic factors are readily obtained in *S. pombe* (Yanagida, 1998), indicating that there is no robust mechanism in this organism to delay or prevent cytokinesis when chromosomes remain in the division plane. Furthermore, the genetic interactions we have uncovered indicate that the CPC promotes, rather than inhibits, cytokinesis in *S. pombe*. In future studies, it will be important to determine whether the sole function of the CPC in *S. pombe* cytokinesis involves regulating Clp1 localization.

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